Original Article

Comparative evaluation of six phenotypic methods for detecting extended-spectrum beta-lactamase-producing *Enterobacteriaceae*

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Abstract

Introduction: Various conventional phenotypic methods and automated systems have been evaluated for extended-spectrum beta-lactamase (ESBL) detection. There is a paucity of data comparing these methods using the same clinical isolates in eastern and north-eastern parts of India. The present study was designed to compare the capacity of six phenotypic methods to detect ESBLs in clinical isolates of *Enterobacteriaceae.*

Methodology: A total of 206 non-duplicate clinical isolates of *Enterobacteriaceae,* obtained over a period of six months (July to December, 2012), were tested by the Vitek 2, double disk synergy tests (30 mm, 20 mm, and modified method), combined disk test, and ESBL Etest to evaluate their ability to detect ESBLs. Minimal inhibitory concentration (MIC) by the agar dilution method was used as the reference method.

Result: The reference method detected ESBLs in 57 (27.7%) isolates. Among the six methods, the combined disk test demonstrated an overall agreement of 100% with the MIC. The Vitek 2 showed a sensitivity and specificity of 91.8% and 97.24%, respectively, with a positive predictive value of 93.33%. The sensitivities of the conventional methods ranged from 83% to 94%. The highest sensitivity and specificity were shown by combined disk (93.44%) and double disk synergy (100%) techniques, respectively.

Conclusion: In our setting, Vitek 2 showed an acceptable capacity to detect ESBL isolates as it improved the turnover time (6 to 8 hours) in comparison to conventional phenotypic methods, which took a minimum of 24 hours. However, the combined disk test achieved the highest sensitivity.

Key words: ESBLs; *Enterobacteriaceae*; Vitek 2

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Introduction

Extended-spectrum β-lactamases (ESBLs), which hydrolyse extended-spectrum cephalosporins and are inhibited by β-lactamase inhibitors such as clavulanic acid, are spreading among *Enterobacteriaceae* [1]. They are usually associated with resistance to multiple unrelated antibiotics such as aminoglycosides, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, and fluoroquinolones, leaving few therapeutic choices [2]. ESBL-producing *Enterobacteriaceae* are now found in ambulatory patients without recognized risk factors for multidrug-resistant organisms [3]. Consequently, recognition of ESBL-producing organisms has become a concern for general hospitals and private practice laboratories. The recent changes in clinical MIC breakpoints for extended-spectrum cephalosporins and for aztreonam against *Enterobacteriaceae* by CLSI and EUCAST decrease the likelihood of interpreting an ESBL-producing *Enterobacteriaceae* as susceptible to extended-spectrum cephalosporins [4,5]. Currently, detection of ESBLs in *Enterobacteriaceae* is still considered useful (CLSI, 2011) or even mandatory (EUCAST, 2011) for epidemiological and infection control purposes [4,5].

Several phenotypic methods have been developed to detect or confirm ESBL production by *Enterobacteriaceae* [6,7,8]. The CLSI issued national guidelines for laboratory detection of *E. coli*, *Proteus mirabilis*, and *Klebsiella* spp. with ESBL [4], but not for species with inducible AmpC β-lactamases, such as *Enterobacter* spp. The Health Protection Agency in the United Kingdom released guidelines for ESBL detection regardless of the tested species [9]. Most guidelines recommend screening isolates based on decreased susceptibility to extended-spectrum cephalosporins in primary susceptibility testing and to use one of the available tests to confirm ESBL production. However, it is not clear which

confirmatory tests are the most sensitive and which extended-spectrum cephalosporins should be tested.

Automated systems are widely used for species identification and susceptibility testing by clinical laboratories to decrease the in-laboratory turnaround time and to improve cost effectiveness. Each system has inherent strengths as well as recognized limitations. Numerous studies have reported on the accuracies and limitations of various automated systems that have forced manufacturers to periodically update their product software [10,11,12].

Various studies comparing different phenotypic methods including automated systems for the detection of ESBLs have been reported across the globe [8,10,11,12,13,14]. No such studies have focused on the eastern and north-eastern parts of India so far. The present study was undertaken to compare the abilities of six phenotypic methods that can be routinely applied in most microbiological laboratories to discriminate between ESBL-positive and negative strains of *Enterobacteriaceae.*

Methodology

Bacterial isolates

A total of 206 non-repetitive isolates of *Enterobacteriaceae* from various clinical samples of urine, blood, pus, wound swab, sputum, or intravenous catheter were obtained from inpatient units of medicine, surgery, gynaecology and obstetrics, pediatrics, and intensive care unit (ICCU) over a period of six months (July to December, 2012). The study included patients of all age groups and both sexes. The samples were processed and isolates were identified following standard laboratory procedures [15].

Detection of ESBLs

Vitek 2 Compact system (bioMérieux, Marcy 1'Étoile, France) [16]

Vitek 2 Compact is an integrated system that automatically performs rapid identification using algorithms based on fluorescence and colorimetry, and antimicrobial susceptibility testing (AST) based on kinetic analysis of growth data. It features an advanced expert system (AES) that interprets the antibiotic resistance patterns, validates the results, and reports the resistance phenotype. A Vitek card for susceptibility testing (AST-GN25), containing ESBL confirming test panel, was inoculated and incubated following the manufacturer's recommendations. An isolate was considered ESBL positive if the phenotypic interpretation by the AES included ESBL with or without decreased outer membrane permeability (*i.e.*, porin loss) and negative if only the wild type or β-lactamases other than ESBLs were proposed by AES. All other interpretation results were considered indeterminate.

Double disk synergy test (30 mm) [17].

A 0.5 McFarland of test isolate was swabbed on a Mueller-Hinton agar plate and 30 μg antibiotic disks of ceftazidime, cefotaxime, cefpodoxime, aztreonam, or cefepime were placed on the plate, 30 mm (center to center) from the amoxicillin/clavulanate (20 μg/10 μg) disk and incubated at 35°C for 18-24 hours. A clear extension of the edge of the antibiotic's inhibition zone toward the disk containing clavulanate was interpreted as synergy, indicating the presence of an ESBL.

Double disk synergy test (20 mm) [6,17,18]

An amoxicillin-clavulanate disk was placed at 20 mm, center to center, of ceftazidime, cefotaxime, cefpodoxime, aztreonam, or cefepime disks on a Mueller-Hinton agar plate. Interpretation criteria for ESBL production were similar as those described above for the double disk synergy test (30 mm).

Modified double disk synergy test [19]

The original double disk synergy test was modified for detecting ESBLs in AmpC-producing isolates. Briefly, a disk of amoxicillin-clavulanate (20/10 μg) or piperacillin-tazobactam (100/10 μg) was placed in the centre of Mueller-Hinton agar; 30 μg disks of cefpodoxime, ceftazidime, cefotaxime, and cefepime were kept at a distance of 20 mm from the amoxycillin/clavulinate disk or piperacillin-tazobactam (center to center). The organisms were considered to be producing ESBL when the zone of inhibition around cefepime or any of the extended-spectrum cephalosporin disks showed a clear-cut increase towards the piperacillin-tazobactam or amoxicillin-clavulanate disks.

Combined disk test [4]

Disks containing 30 μg of cefotaxime, ceftazidime, or cefepime, and disks containing a combination of the three drugs plus 10 μg of clavulanic acid (HiMedia, Mumbai, India) were placed independently, 30 mm apart, on a lawn culture of 0.5 McFarland opacity of the test isolate on a Mueller-Hinton agar plate and incubated for 18-24 hours at 35°C. Isolates were considered ESBL positive if the inhibition zone measured around one of the combination disks after

overnight incubation was at least 5 mm larger than that of the corresponding cephalosporin disk.

ESBL Etest

Three ESBL Etest strips containing cefotaxime/cefotaxime-clavulanic acid (CT/CTL), ceftazidime/ceftazidime-clavulanic acid (TZ/TZL), and cefepime/cefepime-clavulanic acid (PM/PML) (AB Biodisk, Solna, Sweden) for testing the synergy between a gradient of concentrations of either cefotaxime, ceftazidime, or cefepime, respectively, and a fixed concentration of clavulanic acid (4 mg/liter) were tested against each isolate on Mueller-Hinton agar. The respective concentrations ranges were as follows: 0.25 to 16 µg/mL and 0.016 to 1 µg/mL for CT/CTL; 0.5 to 32 µg/mL and 0.064 to 4 µg/mL for TZ/TZL; 0.25 to 16 µg/mL; and, 0.064 to 4 µg/mL for PM/PML. Interpretation criteria followed the manufacturer's recommendations, and isolates were considered ESBL positive when there was (i) a reduction of the MIC by three doubling dilutions in the presence of clavulanic acid (*i.e.*, MIC ratio of ≥ 8) for any of the three cephalosporins, or (ii) a phantom zone or deformation of the cefotaxime, ceftazidime, or cefepime inhibition ellipse at the tapering end regardless of MIC ratios. An isolate was ESBL negative when the MIC ratio was ≤ 8. A result was considered indeterminate when MICs were higher than the predefined range (making it impossible to calculate the MIC ratio) or when one of the tested strip displayed an indeterminate result and the other produced a negative result.

A triple ESBL detection strip (HiMedia, Mumbai, India) containing ceftazidime, cefotxime, and cefepime (0.125-16 µg/mL) in one half, and the other half coated with ceftazidime, cefotxime, and cefepime plus clavulanic acid and tazobactam (0.032-4 µg/mL), was also tested against each isolate. Interpretation was similar to the above criteria for the individual Etest strip.

Reference method [4]

The reference method was MIC by the agar dilution technique performed in accordance with CLSI guidelines. The MIC test was done on all the isolates.

The agar dilution method was performed with Mueller-Hinton agar plates containing serial twofold dilutions of cefotaxime, ceftazidime, and cefepime at concentrations ranging from 0.25 to 512 µg/mL, with and without clavulanic acid at a fixed concentration of 4 µg/mL. Each bacterial suspension was inoculated as spots with a wire loop calibrated to deliver 0.001 mL spread over a small area and incubated at 37°C for 18 to 24 hours. The test was positive if a ≥ 3 twofold reduction was observed in the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone.

Quality control

Klebsiella pneumonia ATCC 700603 and *Escherichia coli* ATCC 25922 were used as ESBL positive and negative controls, respectively.

Statistical analysis [20]

The diagnostic capacity of each phenotypic method was evaluated by analyzing sensitivity, specificity, and positive and negative predictive values. MIC by the agar dilution method was used as the reference standard.

Results

Of the 206 non-repeat strains of *Enterobacteriaceae* that were included in the study, the isolated organisms were *E. coli* (n = 76)*, Klebsiella pneumoniae* (n = 61*), K. oxytoca* (n = 12)*, Proteus mirabilis* (n = 19*), Proteus vulgaris* (n = 11), *Citrobacter freundii* (n = 7)*, Citrobacter koseri (n = 2), Enterobacter cloacae* (n = 6), *Enterobacter aerogenes (n = 2), Salmonella typhi* (n = 4), and *Salmonella paratyphi A* (n = 6). ESBL production was observed in 57 (27.67%) isolates by MIC (agar dilution method) from 206 isolates (Table 1).

Vitek 2 system

The Vitek 2 method detected 56 out of 57 ESBL strains, resulting in a sensitivity of 91.8% (Tables 2, 3). This method showed the maximum number of false positives (n = 4) (Table 2).

Double disk synergy test (30 mm)

By using the double disk synergy test at 30 mm, sensitivity reached 83.61%. Cefepime yielded the highest performance among the five β-lactams, with a sensitivity and specificity of 81.97% and 100%, respectively (Table 3). The sensitivity was improved when taking into account the results obtained with the combination of two of the five β-lactams, with a higher sensitivity obtained when testing cefotaxime and cefepime (sensitivity 83.6% and specificity 100%). The maximum number of false negatives (n = 10) was observed in this method (Table 2).

Table 1. Distribution of ESBL among the *Enterobacteriaceae* species

Enterobacteriaceae species	No. of isolates	ESBL positive by MIC (%)
E. coli	76	26 (34.2)
Klebsiella. pneumoniae	61	16 (26.22)
K. oxytoca	12	3 (25)
Proteus mirabilis	19	5 (55.55)
Proteus vulgaris	11	2 (18.18)
Salmonella typhi	4	1 (25)
Salmonella paratyphi A	6	1 (16.66)
Citrobacter freundii	7	1 (14.28)
Citrobacter koseri	2	0
Enterobacter cloacae	6	1 (16.66)
Enterobacter aerogenes	2	1 (50)
Total	206	57 (27.67)

ESBL: extended-spectrum beta-lactamase; MIC: minimal inhibitory concentration

Table 2. Distribution of ESBL isolates among the phenotypic methods

Reference method		No. of ESBL isolates		Total
Phenotypic methods		Positive	Negative	
1. Vitek 2	Positive	56	4	60
	Negative	5	141	146
2. DDST (30 mm)	Positive	51	0	51
	Negative	10	145	155
3. DDST (20 mm)	Positive	54	0	54
	Negative	7	145	152
4. MDDST	Positive	56	0	56
	Negative	5	145	150
5. CDT	Positive	57	1	58
	Negative	4	144	148
6. ESBL Etest	Positive	56	1	57
	Negative	5	144	149

ESBL: extended-spectrum beta-lactamase; DDST: double disk synergy test; MDDST: modified double disk synergy test; CDT: combined disk test

Table 3. Statistical analysis of the various parameters of six phenotypic methods

Phenotypic methods	All isolates (206)			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Vitek 2				
AST-GN25	91.8	97.2	93.33	96.58
Double disk synergy test (30 mm)				
Ceftazidime	78.68	100	100	91.77
Cefotaxime	80.33	100	100	92.35
Cefpodoxime	73.77	100	100	90
Aztreonam	70.49	100	100	88.95
Cefepime	81.97	100	100	92.94
Ceftazidime + cefotaxime	81.97	100	100	92.94
Ceftazidime + aztreonam	75.4	100	100	90.62
Ceftazidime + cefepime	83.6	100	100	92.94
Cefotaxime + aztreonam	81.97	100	100	92.94
Cefotaxime + cefepime	83.6	100	100	93.54
Double disk synergy test (20 mm)				
Ceftazidime	81.86	100	100	92.94
Cefotaxime	83.6	100	100	93.54
Cefpodoxime	77	100	100	91.19
Aztreonam	72	100	100	89.5
Cefepime	86.88	100	100	94.77
Ceftazidime + cefotaxime	85.24	100	100	94.15
Ceftazidime + aztreonam	80.32	100	100	92.35
Ceftazidime + cefepime	86.88	100	100	94.77
Cefotaxime + aztreonam	85.24	100	100	94.15
Cefotaxime + cefepime	88.52	100	100	95.39
Modified double disk synergy test (20 mm)				
Cefpodoxime, ceftazidime, cefotaxime & cefepime with amoxicillin/clavulanate at center	86.88	100	100	94.77
Cefpodoxime, ceftazidime, cefotaxime & cefepime with pipercillin/tazobactam at center	91.8	100	100	96.66
Combined disk test				
Ceftazidime & ceftazidime/clavulanate	88.52	100	100	95.39
Cefotaxime & cefotaxime/clavulanate	91.8	99.31	98.24	96.64
Cefepime & cefepime/clavulanate	93.44	99.31	98.27	97.29
Ceftazidime & ceftazidime/clavulanate + cefepime & cefepime/clavulanate	90.16	100	100	96.02
Cefotaxime & cefotaxime/clavulanate + cefepime & cefepime/clavulanate	93.44	99.33	98.27	97.29
ESBL Etest				
Ceftazidime	83.6	100	100	93.54
Cefotaxime	88.52	100	100	95.39
Cefepime	91.8	99.31	98.24	96.64
Cefotaxime + ceftazidime + cefepime	91.8	99.31	98.24	96.64

PPV: positive predictive value; NPV: negative predictive value

Double disk synergy test (20 mm)

Sensitivity achieved 86.88% for cefepime when the distance was kept at 20 mm apart, but was lower for the other four β-lactams tested. However, the combination of two disks increased the sensitivity; the highest (88.52%) was observed with cefotaxime and cefepime compared to the other combinations tested (Table 3).

Modified double disk synergy test (20 mm)

This method identified 53 out of 57 ESBL isolates. When amoxicillin-clavulanate was kept at the centre along with pipercillin-tazobactam, 56 isolates were determined to be ESBLs, resulting in a sensitivity of 91.8% (Table 3).

Combined disk method

Among the six methods used, the combined disk test detected all the ESBL isolates (n = 57). It was able to pick up one more isolate of *S. Typhi* as ESBL that other methods failed to identify. Using this technique, cefepime with cefepime-clavulanate achieved the highest sensitivity of 93.44%, and ceftazidime with ceftazidime-clavulanate the highest specificity (100%) (Table 3).

ESBL Etests

The highest sensitivity (91.8%) was obtained with cefepime. However, using the combination strip of cefotaxime, ceftazidime, and cefepime did not increase sensitivity and specificity.

Statistical comparisons

Since the main goal of ESBL detection is to achieve high sensitivity, statistical comparisons were evaluated among the six methods. The Vitek 2 had significantly higher sensitivity than both the double disk synergy test (30 mm, 20 mm) and the modified disk synergy test, but lower sensitivity than the combined disk method. The double disk synergy (30 mm & 20 mm), including modified disk synergy tests, had significantly higher specificities than other tests (Table 3).

Discussion

The Vitek 2 system's ability to detect ESBL production was rather low, with a sensitivity of 92% to 95% and specificity of 50% to 79% in *E. coli* and *K. pneumoniae* [7,14,21]. In our study, this method showed a sensitivity and specificity of 91.8% and 97.2%, respectively. Microbiologists should keep in mind that this technique has been validated only for few species, such as *E. coli, K. pneumoniae, K.oxytoca, Proteus mirabilis, Proteus vulgaris*, and that it is not reliable in detecting ESBL among other species of *Enterobacteriaceae*, although some authors have reported a high sensitivity in combination with very low specificity [7].

The high sensitivity of the disk diffusion method when using two or more extended-spectrum cephalosporins has been previously reported [7,22]. In the present study, the combination of cefotaxime and ceftazidime achieved 81.97% sensitivity to adequately detect ESBL production when a distance of 30 mm was maintained between the amoxicillin-clavulanic acid and cephalosporins disks, but the inclusion of cefepime increased the sensitivity to 83.6%. However, by decreasing the distance between the disks (center to center) to 20 mm, the sensitivity of the cefotaxime and ceftazidime combination increased to 85%, and further improved to 88.5% with the combination of cefepime. To overcome the problem of optimal disk spacing, Thomson and Sanders used the recommended disk spacing of 30 mm and then repeated at 20 mm to see if the former disk spacing was negative [6].

The modified double disk synergy method was reported previously to increase the sensitivity of the double disk method [6,17,23]. We observed a sensitivity of 91.8% with this method when piperacillin-tazobactam was kept at the center and a distance of 20 mm was maintained between the disks. It has been reported that clavulanic acid may induce expression of high levels of AmpC production in organisms producing both ESBL and AmpC together, and may antagonize rather than protect the antibacterial activity of the partner β-lactam, thereby masking any synergy arising from inhibition of an ESBL. Much better inhibition is achieved with the sulphones, such as tazobactam and sulbactam, which are preferable inhibitors for ESBL detection tests in AmpC producers [24,25].

The ability of the combined disk method to detect ESBL is very satisfactory, and sensitivity can reach 100% when testing both cefotaxime and cefepime [26]. Another report showed that the sensitivity after testing the two latter drugs was not different from that of cefotaxime alone [14]. The present study demonstrated that this method achieved the highest sensitivity (93.44%) among all the phenotypic tests applied. However, in our setting, sensitivity remained the same even with the combination of cefotaxime and cefepime.

The manufacturer recommended to test cefotaxime and ceftazidime as the first-line method with ESBL

Etest strips and to complete testing with the cefepime ESBL Etest in cases with an inconclusive result from the first two strips. Among the four ESBL Etest strips used in our study, the sensitivity of cefepime (91.8%) and the combination strip of ceftazidime, ceotaxime and cefepime (91.8%) were significantly higher than those obtained with cefotaxime (83.6%) and ceftazidime (88.52%). Such a high sensitivity of the cefepime was previously reported by Wiegand *et al*. and Sturenberg *et al*. [7,27].

In our setting, cefepime was the most effective cephalosporin in detecting ESBL producers; it was followed by cefotaxime, ceftazidime, cefpodoxime, and aztreonam. Similar findings were observed by Sturenberg *et al*. and Garrec *et al*. [14,27]. However, Cormican *et al*. showed maximum ESBL detection by ceftazidime [28]. The combined disk method was the most accurate for detecting ESBLs, as it showed 100% agreement with the reference method. The Vitek 2, double disk synergy (30 mm), double disk synergy (20 mm), modified double disk synergy method, and ESBL Etest demonstrated 98.2%, 89.5%, 94.7%, 98.2%, and 98.2% concordant result with the gold standard, respectively. Stefaniuk *et al*. reported that Vitek 2 showed 94% agreement with the reference method (MIC agar dilution) [29].

The limitation of this study was that PCR could not be used as the gold standard due to its unavailability in our institute. Instead, we applied MIC by the agar dilution technique as the reference method for our study. We also could not employ the modified CLSI combined disk method reported by Tsakri *et al*. as boronic acid could not be acquired [30].

In conclusion, considering the challenging nature of the isolates, the six phenotypic methods were highly sensitive and specific at ESBL detection, with the combined disk test (93.44% sensitivity) being the most sensitive. For rapid detection, Vitek 2 was the better choice, as the other methods took at least 24 hours to produce the final result. The only limitation of the Vitek 2 in developing countries is the high cost of the system and its identification and antibiotic susceptibility testing cards.

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